Sources, isolation, characterisation and evaluation of probiotics

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Abstract

Probiotics are live microorganisms that, when ingested in adequate amounts, provide health benefits to the host. The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria, which are isolated from traditional fermented products and the gut, faeces and breast milk of human subjects. The identification of microorganisms is the first step in the selection of potential probiotics. The present techniques, including genetic fingerprinting, gene sequencing, oligonucleotide probes and specific primer selection, discriminate closely related bacteria with varying degrees of success. Additional molecular methods, such as denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis and fluorescence *in situ* hybridisation, are employed to identify and characterise probiotics. The ability to examine fully sequenced genomes has accelerated the application of genetic approaches to elucidate the functional roles of probiotics. One of the best-demonstrated clinical benefits of probiotics is the prevention and treatment of acute and antibiotic-associated diarrhoea; however, there is mounting evidence for a potential role for probiotics in the treatment of allergies and intestinal, liver and metabolic diseases. These positive effects are generally attributed to the ability of probiotics to regulate intestinal permeability, normalise host intestinal microbiota, improve gut immune barrier function and equilibrate the balance between pro-inflammatory and anti-inflammatory cytokines. However, the positive effects of probiotics are not always substantiated by findings from properly conducted clinical trials. Notably, even when the results from randomised, placebo-controlled trials support the beneficial effects of a particular probiotic for a specific indication, the benefits are generally not translatable to other probiotic formulations.

Key words: Bifidobacteria: Lactic acid bacteria: Lactobacilli: Probiotics: Diseases

Currently, there is an increasing interest in and demand for probiotics, after a long history of safe use in fermented dairy products and an increased recognition of the beneficial effects of probiotics to human gut health[1]. According to the FAO of the UN and the WHO[2], probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus*, the predominant and sub-dominant groups of the gastrointestinal microbiota, respectively[3], are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements[4–6]. The yeast *Saccharomyces boulardii* has also been shown to have health benefits[7].

For probiotics to be successful, they must possess certain characteristics. The criteria for the selection of probiotics include tolerance to gastrointestinal conditions (gastric acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens[8,9]. Traditionally, it has been proposed that a useful probiotic must fulfil the following criteria:

(1) Have a demonstrated beneficial effect on the host.
(2) Be non-pathogenic, non-toxic and free of significant adverse side effects.
(3) Be able to survive through the gastrointestinal tract (GIT; *in vitro* and *in vivo*).
(4) Be present in the product in an adequate number of viable cells to confer the health benefit.
(5) Be compatible with product matrix, processing and storage conditions to maintain the desired properties, and labelled accurately[10].

The results of evidence-based analyses from human studies and animal models have shown the potential clinical effectiveness of probiotics on many diseases[11]. In fact, probiotics have been reported to suppress diarrhoea[12], alleviate lactose intolerance[13] and post-operative complications[14], exhibit antimicrobial[15] and anti-colorectal cancer activities[16,17], reduce irritable bowel symptoms[18] and prevent inflammatory bowel disease[19]. However, generalisations concerning the potential health benefits of probiotics should be not made

because probiotic effects tend to be strain specific; thus, the health benefit attributed to one strain is not necessarily applicable to another strain, even within one species[20].

The mechanisms underlying the beneficial effects of probiotics are largely unknown but are likely to be multi-factorial. However, several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include modification of the gut microbiota, competitive adherence to the mucosa and the epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host.

The aim of the present work was to review the sources, isolation methodology, characterisation and evaluation of probiotic strains. The various steps needed to characterise a bacterial strain as a novel probiotic are depicted in Fig. 1.

In the present study, we sought to conduct a literature review of the sources, isolation and characterisation and evaluation of probiotic strains. The present review summarises a total of 1500 works, published to the date from PubMed database (February 2012), and intends to provide an historical context and the state of this field. For this aim, the following search combinations were used: probiotics and sources; lactobacillus and isolation; bifidobacteria and isolation; probiotics and breast milk; probiotics and origin probiotics and fermented foods; isolation and characterisation and probiotics; probiotics and evaluation; and probiotics and randomised controlled trial (RCT).

Sources

Dairy and dairy-related products are a good source of probiotics[1]. Within this context, lactic acid bacteria (LAB), bifidobacteria and other microorganisms obtained from fermented milks have been used for centuries. Spontaneous milk fermentation has a long history in different regions of Mongolia or Africa, and the use of beneficial microorganisms in fermented dairy products has been practised for many generations[21].
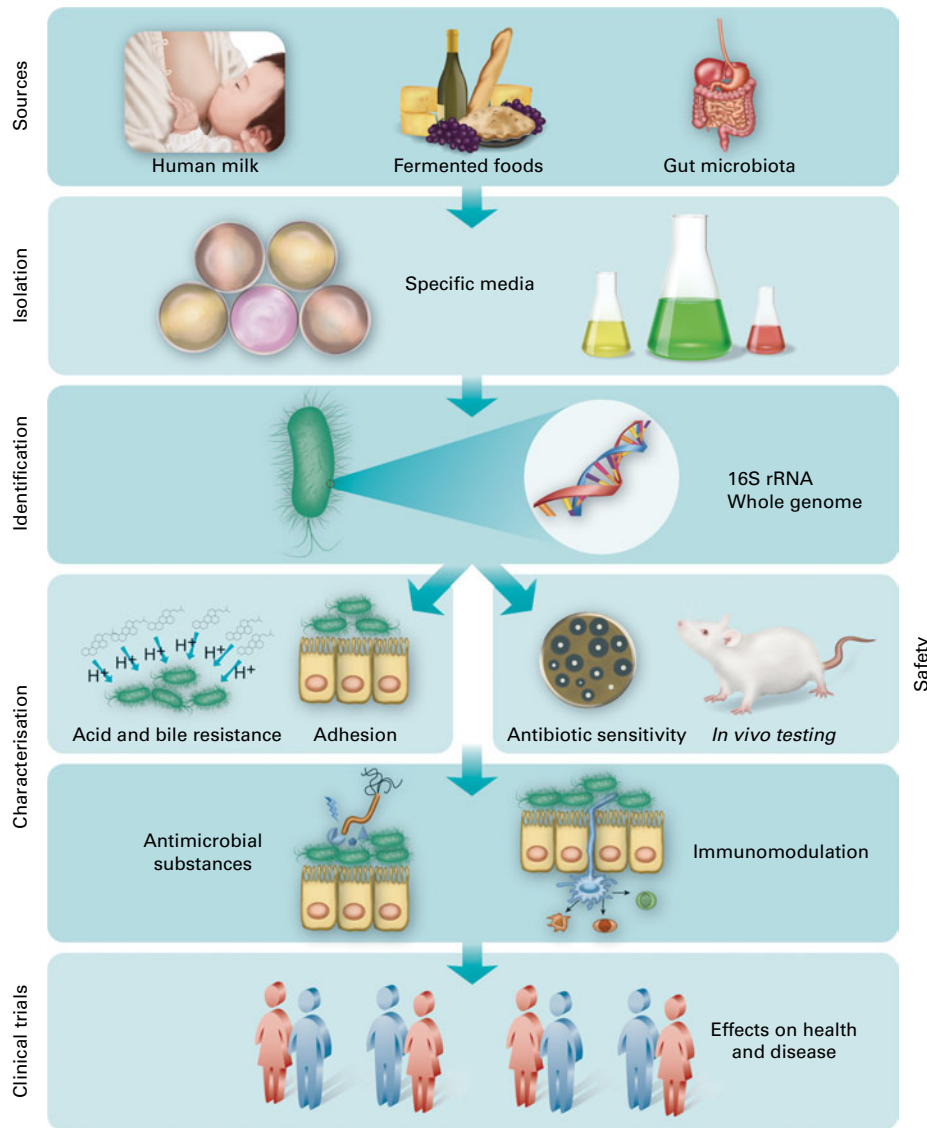

Fig. 1. Flow chart describing the various steps to be followed in order for a bacterial strain to qualify as a novel probiotic. rRNA, ribosomal RNA.

These traditional fermented milks contain complex compositions of LAB species and therefore provide a useful source of probiotic strains. Thus, it is not surprising that in a recent work, 148 LAB strains were isolated from Kurut, a traditional naturally fermented yak milk from China in which *L. delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus* are the predominant microbial populations[22]. In addition, yeasts and lactobacillus strains with probiotic properties have been isolated from kefir grains, Masai milk and Koumiss, a fermented milk drink; these microorganisms are able to influence immune responses[23–26].

Recent studies were conducted to evaluate traditional fermented products as potential natural sources of probiotic bacteria. Generally, most of the microorganisms isolated from fermented products belong to the *Lactobacillus* genus[27–29]. Interestingly, in a recent work, a *Weisella* strain was isolated from Nigerian fermented foods and selected for its probiotic potential[30].

Cheese is a dairy product with potential for the delivery of probiotic microorganisms into the human intestine. *L. plantarum* strains have been isolated from Italian, Argentinean and Bulgarian cheeses[31–33].

Interestingly, it was observed that breast milk is not sterile, even when collected aseptically, which raises the possibility that breast milk harbours a natural bacterial inoculum[34]. The bacteria in breast milk have long been considered to be a consequence of skin or faecal contamination. Although the lactobacilli present in human milk are genotypically different from those isolated from the skin[35,36], and the LAB strains present in breast milk were also observed in the faeces of the corresponding infants[37], it has only recently become accepted that breast milk constitutes an interesting source of probiotic LAB and bifidobacteria for inclusion in infant formulas and foods targeted to both pre-term and full-term infants[38]. In addition, it has been reported that breast-fed infants have fewer allergies and gastrointestinal infections than formula-fed infants; therefore, the intestinal microbiota in the breast-fed infant might be considered to be ideally healthy[39]. Human breast milk comprises several predominant bacteria, such as staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci and bifidobacteria[36,37,39–42], and its intake favours the predominance of bifidobacteria and lactobacilli in the infant intestinal microbiota. Several authors have reported that lactobacilli isolated from breast milk are an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation[43,44]. Moreover, it was reported that two *Lactobacillus* strains isolated from human breast milk enhanced natural and acquired immune responses through the activation of the natural killer and T-cell subsets and the expansion of regulatory T cells[42].

Another source of probiotics is the human GIT. More than 500 different bacterial species reside in the adult human gut. In fact, many of the probiotic strains used today have been isolated from this source, such as *L. gasseri* and *L. reuteri*[45]. In addition, it has been reported that *L. fermentum*, isolated from human colonic mucosal biopsy samples, possesses antimicrobial activities against food-borne pathogens[46]. A common misconception is that probiotics must always colonise the intestinal tract to exert their effects. In fact, certain probiotics (e.g. *B. longum* and *Bacteroides thetaiotaomicron*) reside in the human intestinal microbiota, but others (e.g. *L. casei* and *B. animalis*) do not[47]. Most of the probiotic strains, such as *B. longum*[48] and *L. acidophilus* RY2[49], were isolated from the faecal samples of healthy adults and infants, respectively. Notably, in concordance with breast milk, several studies have reported the isolation of probiotics from breast-fed infant faeces[50,51].

The isolation of probiotics is not limited to the human tract. The guts of several animal species, including pigs, rats and even poultry, are good sources of probiotics[52]. Recently, *L. johnsonii* CRL 1647, isolated from the *Apis mellifera* L. bee gut, was shown to exhibit a beneficial effect on honeybee colonies[53]. Additionally, probiotic strains have been obtained from the intestinal tracts of marine and freshwater fish, such as *Carassius auratus gibelio*[54], rainbow trout[55] or shrimp[56].

Other studies show that probiotic strains are also found in non-dairy fermented substrates[57]. *In vitro* experiments have demonstrated that certain bacterial strains, isolated from meat (*L. sakei*, *L. curvatus* and *Staphylococcus carnosus*) and fruits (*L. paracasei* and *L. plantarum*), can display functional and metabolic properties similar to those of human intestinal bacteria[58]. In addition, a recent work described the isolation of a *Lactobacillus* strain from brines of naturally fermented Aloreña green table olives[59]. Moreover, *L. buchneri* P2, isolated from pickled juice, demonstrated probiotic properties, such as cholesterol reduction, acid and bile tolerance and antimicrobial activity[60].

Isolation, identification, characterisation and safety

In microbial ecology, it is generally accepted that cultivation-based approaches provide an incomplete picture of microbial diversity. Ecological niches present a complex interrelation between the different species of microbes that cannot be mimicked using traditional culture methods. Molecular approaches that bypass the cultivation step have become popular as a means of identifying the microbial diversity of different sources. These methods have provided important information concerning microbial ecosystems, including the sources of probiotics. The first important step in studying an ecosystem is the isolation of its members. The identification of the microbes, especially in probiotic bacteria, is not valuable when we want to determine *in vivo* functions associated with beneficial effects on human health.

Isolation

The first step in the isolation of probiotic bacteria is to maintain the sample in adequate conditions before incubation in selective media. Most probiotics are anaerobic or facultatively anaerobic; therefore, the samples should be immediately placed under anaerobic conditions and processed as soon as possible (within 3 h). The samples should be homogenised quickly and diluted and cultured in selective media (Sergio Muñoz-Quezada, Empar Chenoll, Jose Maria Vieites, Salvador Genoves, Jose Maldonado, Miriam Bermudez-Brito, Carolina Gomez-Llorente, Esther Matencio, Maria Jose Bernal, Fernando Romero, Antonio Suárez, Daniel Ramon, Angel Gil, unpublished results).

Several media have been devised for the elective or selective isolation of bifidobacteria and lactobacilli[61–70]. Rogosa *et al.*[68] developed a selective medium for the isolation and enumeration of oral and faecal lactobacilli and *Bifidobacterium* that contains a Columbia agar base supplemented with propionic acid. The low pH of this medium, which is tolerated by lactobacilli and bifidobacteria, inhibits the growth of other predominating organisms in human faeces, such as *Bacteroides* and *Eubacterium* species.

The plates are incubated at 37°C for 48–72 h in an anaerobic atmosphere for the growth of bifidobacteria and other anaerobic species or in a CO_2-rich atmosphere for the growth of lactobacilli. Subsequently, the colonies are isolated and transferred to broth or a new agar plate.

Identification

The identification of microbes in the GIT or food sources is the first step in the selection of potential probiotics. For many ecosystems, only a small percentage of microbes can be grown in culture[71]. The taxonomic classification might be defined as the process of cataloguing biodiversity based on a polyphasic approach[72], which involves genotypic and phenotypic methods. Historically, phenotypic methods have been used to identify bacteria. The taxonomy for many decades heavily relied on the type of sugar fermentation and the fermentation products generated. Thus, the probiotics have been primarily classified as LAB. Today, 16S RNA gene analysis has become the method of choice. For the past two decades, microbiologists have used this conserved fragment for phylogenetic classification[73,74], and the relatedness among organisms is estimated through the comparison of their sequences in available databases (DDBJ, ENA, GenBank)[75–77]. The 16S RNA gene analysis has been combined with other methods to identify bacterial communities of the gut or ecological sources. The amplified 16S DNA can be coupled with PAGE using temperature (temperature gradient gel electrophoresis) or chemical denaturation (denaturing gradient gel electrophoresis)[78], hybridised using fluorescent oligonucleotide probes that target specific 16S (fluorescence *in situ* hybridisation)[79,80] or digested with restriction enzymes (Terminal restriction fragment length polymorphism (T-RFLP)).

However, the 16S DNA fragment is extremely small (1500 bp) compared with the bacterial genome (30 000–40 000 bp). Complementary information is typically necessary due to insufficient base sequence diversity to differentiate strains of a given species. The 16S to 23S intergenic spacer region exhibits a great deal of sequence and length variation[81]. The variation in this region has been used for differentiating species of prokaryotes. Undoubtedly, the analysis of the bacterial genome is the most useful tool to identify and characterise the processes underlying speciation and evolution in prokaryotes[82]. However, genome sequencing remains a laborious and relatively expensive technique.

Characterisation

The species of the genera *Lactobacillus* and *Bifidobacterium* are among the most important taxa of probiotics. When ingested, sufficient numbers of metabolically active bacteria must overcome the GIT barrier and transitorily persist in the GIT to exert their beneficial effects. This characteristic is important, although certain authors have shown beneficial effects of dead probiotics[83]. The capacity to tolerate an extremely low pH (1·5–3·0), gastric enzymes, bile salts and other intestinal enzymes, are the challenges for arriving alive in the GIT[84]. Various *in vitro* assays have been designed to mimic these stress conditions.

Resistance to low pH and biliary salts. Acid tolerance is one of the general criteria that is considered during the selection of potential probiotic strains to guarantee their viability and functionality[85]. *In vitro* systems, including controlled incubations in real or simulated gastric juices (pH 2·0–4·0; 70–180 min[86]), have been preferentially used in the evaluation of new probiotic strains. Complex models that simulate gastrointestinal transit have been developed[84,87]. Moreover, 1–4 h incubations in chemical and/or enzymatic media at a pH range of 1·5–3·0 have also been performed.

The biliary salts facilitate the digestion of lipophilic compounds, but also behave as an antimicrobial agent by directly influencing the establishment of the intestinal microbiota. The relevant physiological concentrations of human bile range from 0·3 to 0·5%[88,89]. *In vitro* assays are conducted in 0·3–0·7% bovine bile (Oxgall) for 60–180 min. Probiotics show highly variable resistance to acid and bile salts, and this characteristic is both species and strain dependent. Several studies have reported that bifidobacteria are highly sensitive to low pH values. Certain species exhibit survival rates of 0% at pH 2·0 for 90 min[86,90], less than 1% at pH 3·0 for 2 h[91] or increased percentages at pH 3·0–5·0 for 3 h[92]. The highest survival rates have been described for certain bifidobacteria[93–96]. Several *Lactobacillus* strains have shown a high resistance to low pH. A study involving twenty *Lactobacillus* strains reported survival rates of 2–100% at pH 3·0 for 1 h. Certain bifidobacteria demonstrated a survival rate of 1–70% in 0·3% Oxgall for 90 min[87]. A total of two *L. plantarum* strains showed greater than 50% survival at pH 2·0 and 3·0 and 1·0% survival in 73–180% bile salt[97].

Bacteria develop an adaptive response under moderate stress conditions, such as nutrient-rich or nutrient-poor media, pH and salt content[98]. Surprisingly, the modulation of protein complexes, transduction of signals or induction of genes[99] might be used to modify food features[100].

Adherence to intestinal epithelial cells. The adherence to intestinal epithelial cells and/or mucus is an important characteristic of probiotics to promote the gut residence time, pathogen exclusion and host and immune system interactions. Over the past 25 years, the Caco-2 cell line has been used extensively to determine adhesion capacity[101]. Caco-2 cells form a homogeneous monolayer that resembles that of human mature enterocytes in the small intestine[102]; they also form crypts, which are typical structures of the epithelial monolayer[103]. The colonic cell line HT-29 also displays typical characteristics of enterocyte differentiation and has been used for *in vitro* adhesion assays[104]. Adhesion to the intestinal mucosa is based on the immobilisation of mucin bound to the surface of microwell plates[105,106] in several commercially

available *in vitro* assays, whereas other useful *in vitro* models utilise cell lines developed to simulate a mucus-secreting environment (HT-29-MTX)[107,108]. The results of *in vitro* adhesion models, cell lines or their combination are highly variable[109]. In fact, lactobacilli, bifidobacteria and pathogens show differences in adhesion to mucus, Caco-2, Caco-2 plus mucus, HT-29 MTX and Caco-2/HT29MTX. For *L. rhamnosus* GG, the reported capacities for adhesion in those systems are 10·21, 5·17, 3·19, 0·84 and 0·85 %, respectively. Several *in vitro* studies have evaluated the adhesion of potential probiotic bacteria and their interactions with pathogens at the intestinal epithelial interface, and the results were dependent on the technique and strains used[105].

Differences in the experimental conditions for assays of acid tolerance (medium acidified using HCl or lactic acid, with or without enzymes), bile resistance (bile origin and dose) and adhesion (mucus, cell lines, cells plus mucus) make it extremely difficult to compare their results. Remarkably, these characteristics are strain-specific traits that can be extremely variable within the species or genus. Therefore, the use of *in vitro* models is necessary to select the most promising strains. Thus, human clinical trials are the definitive tool to establish probiotic functionality.

Antimicrobial activity. When administered in adequate amounts, probiotics confer health benefits to the host[85]. An important beneficial effect is antimicrobial activity against pathogens[109]. Probiotics might act through a variety of mechanisms, including the production of antimicrobial substances, competition with pathogens for nutrients and adhesion sites and stimulation of the immune system[110].

Intestinal infections are mediated by the adhesion of pathogenic bacteria to mucosal surfaces and disruption of the intestinal microbiota. The probiotic bacteria might play protective roles through adhesion and colonisation of the mucosal surfaces, effectively competing with pathogens for binding sites and nutrients or/and immune stimulation[111,112]. Ferreira *et al.*[113] evaluated the ability of seven newly isolated strains of *L. gasseri* to adhere to intestinal mucosa and to auto-aggregate and co-aggregate with the model pathogens *Cronobacter sakazakii* (ATCC 29 544) and *Clostridium difficile* (ATCC 1296). All of the viable and non-viable bacterial strains used alone or in combination were able to auto-aggregate. The co-aggregation with *C. sakazakii* or *C. difficile* was higher ($P < 0.05$) in the non-viable bacterial strains.

The ability of probiotic strains to inhibit the growth of pathogens in broth and agar plates and to modulate the production of cytokines and growth factors in cell lines has been well documented using *in vitro* models in the evaluation of their biological effects. In addition, mice and other animal models are also useful to study the antimicrobial activity of probiotics. The antimicrobial effects of novel probiotics have been tested against *Listeria monocytogenes* and *Helicobacter pylori in vitro*, and against human rotavirus using *in vivo* infection models[94,95]. Several strains of lactobacilli and bifidobacteria successfully inhibited the growth of *Escherichia coli*[104,114–117], *Salmonella typhimurium*, *Shigella flexneri*[118–120] and *C. difficile*[121]. Moreover, an *L. plantarum* strain produced compounds with antifungal activity[122].

Notably, in these studies, single strains were tested and the antimicrobial activities in most cases were due to the mixed host immune modulation and anti-infective activity of probiotics.

Although, in clinical studies, the use of probiotics is promising for the management of diarrhoea, *H. pylori* infection, atopic disease, necrotising enterocolitis (NEC) and inflammatory bowel diseases, there are uncertainties concerning the relative significance of probiotics, and the results from meta-analyses to determine the beneficial effects of probiotics[123] are contradictory. Moreover, individual or mixtures of probiotic strains and the required dosages need to be determined, and additional clinical trials should be conducted to improve the available information.

Safety

Detailed reviews and opinions of present practices in the safety assessment of probiotics for human subjects have been published[124,125]. The European Food Safety Authority was established in 2002 to address the increasingly important and complex scientific and technical issues concerning food and feed safety in the European Union (regulation no. 178/2002), but no formal safety testing guidelines for food-associated microbes have been established. The Scientific Committee on Animal Nutrition proposed the 'qualified presumption of safety'[126] as an approach to safety evaluation, which involves four steps: (1) defining the taxonomy of the microbe; (2) collecting sufficient information providing the basis for qualified presumption of safety status, including scientific literature, history of use, industrial applications and ecological and human intervention data; (3) excluding pathogenicity and (4) defining the end use. If there are no safety concerns for a certain taxonomic group, or if any safety concerns have been allayed (qualification), then qualified presumption of safety status may be granted[127].

The LAB will be among the first groups to be evaluated. The introduction of this system appears to be favourably received and is considered to be more flexible than the Generally Recognised As Safe system used in the United States because it considers new emerging safety risks, such as the acquisition of antibiotic resistance and virulence determinants.

A variety of factors are considered in the assessment of the safety of probiotics, which include the following: (1) recording the isolation history and taxonomic classification of candidate probiotics, (2) manufacturing controls that eliminate contamination (including cross-contamination between batches) of probiotics with microbes or other substances, (3) assessing the association of probiotics with infectivity or toxicity at the strain level and (4) determining the physiological status of the consuming population, with special consideration for use in vulnerable populations, including newborn infants and the critically ill (dose administered and method of administration). When considering all of these factors, probiotics are generally considered to be 'safe', but this assumption cannot be made broadly, and such an assessment is specific to the many conditions indicated earlier. To market probiotics as

foods or dietary supplements, the safety of each particular strain for the general population[128] needs to be determined.

Industrial production of probiotics

The next step after a probiotic strain has been isolated, identified and characterised, and its safety has been approved, is scale-up production. Industrial production relies on two aspects. First, the microorganism needs to be cultured in adequate medium to allow growth in large quantities. Second, probiotic viability during manufacturing has to be secured. Both aspects are important, and scale-up production may become a bottleneck for an initially promising microorganism. Thus, certain strains might not grow properly, stand freeze- or spray-drying processes, or addition of preservatives to maintain viability throughout the shelf-life of the manufactured product.

Evaluation of probiotics

Preclinical evaluation

There is substantial evidence from *in vitro* and animal studies that known and potential probiotics exhibit strain-specific immunomodulatory effects.

In vitro studies. A large inventory of animal and human cell lines is available as models of the gut[129], such as Caco-2, HT-29, IEC-6, IEC-18 and T84, to name a few. In most of the *in vitro* experimental models, the epithelial cells are cultivated as monolayers in which the establishment of a functional epithelial feature is not achieved.

To overcome this problem, researchers have attempted to reconcile the mechanisms underlying the complex and dynamic interactions between the intestinal epithelium and bacteria on the luminal side, and the epithelium and cells of the immune system on the basolateral side, using co-culture experiments with probiotics, dendritic cells, intestinal epithelial cells[130] and 3D models[129]. The 3D models are generated using an intestinal epithelial cell line of non-carcinogenic origin, which is cultured on a microporous membrane, enabling the polarisation of the cells. Below the microporous membrane (basolateral side), the epithelial cells are underlaid with immune cells (macrophages, dendritic cells), mimicking mucosal lymphoid tissue. Intestinal microbiota are added to the apical side of the membrane to study the effects of the microbiota. These three components (epithelia, immune cells and microbiota) are the most important factors in the gut; therefore, these models closely mimic the *in vivo* situation.

Animal studies. The immunomodulatory effects of probiotics have been demonstrated in experimental models of allergy, autoimmunity and inflammatory bowel disease[130]. Probiotic supplementation has exhibited protective effects during spontaneous and chemically induced colitis by down-regulating the production of inflammatory cytokines or inducing regulatory mechanisms in a strain-specific manner. In animal models of allergen sensitisation and murine models of asthma and allergic rhinitis (AR), orally administered probiotics have demonstrated a strain-dependent decrease in IgE production by modulating systemic cytokine production. Certain probiotics have been shown to decrease airway hyper-responsiveness and inflammation through the induction of regulatory mechanisms.

Clinical evaluation

Many clinical studies have attempted to evaluate a great variety of probiotics under diverse physiological conditions and pathologies. However, many of these studies are flawed due to the small number of patients used or the lack of a control group. In fact, the European Food Safety Authority delivers scientific opinions on the substantiation of health claims related to probiotic strains. A high percentage of these claims is rejected by the European Food Safety Authority because a cause and effect relationship is not clearly established between the consumption of the probiotic and the beneficial effect it is supposed to have (mostly due to the small number, or even lack, of human intervention studies demonstrating such effects). The most reliable method of assessing the therapeutic benefits of any probiotic strain is the use of randomised, placebo-controlled trials, which are reviewed later and appear in Table 1.

Pregnancy and lactation. Asemi *et al.*[131] assessed the effects of the daily consumption of probiotic yoghurt on inflammatory factors in pregnant women. The subjects consumed 200 g of probiotic yoghurt containing *L. acidophilus* La5 and *B. animalis* BB12 or 200 g of conventional yoghurt daily for 9 weeks. The consumption of the probiotic yoghurt significantly decreased the expression of C-reactive protein, but had no effect on TNF-α in these subjects. In addition, the consumption of probiotic yoghurt among pregnant women resulted in increased levels of erythrocyte glutathione reductase but did not affect other indices of oxidative stress.[132]

Dugoua *et al.*[133] reported that *Lactobacillus* and *Bifidobacterium* had no effect on the incidence of Caesarean section, birth weight or gestational age.

As mentioned in the 'Sources' section of the present review, lactobacilli isolated from breast milk are an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation[43,44]

Allergy. Vliagoftis *et al.*[134] evaluated the clinical evidence for the use of probiotics as a therapeutic modality for AR and asthma. The review included twelve RCT. A total of nine trials that evaluated clinical outcomes in AR showed an improvement due to the use of probiotics. All of the trials concerning perennial AR showed reduced symptom scoring and medication use with the administration of probiotics compared with the placebo. Moreover, in the five trials concerning seasonal AR, an improvement in the clinical outcomes was shown. The nine studies that reported various immunologic measurements of allergy showed no significant probiotic effects. The trials concerning the effect of probiotic administration on the treatment of asthma showed no positive effects. Taken together, these results suggest that probiotics might

Table 1. Summary of selected human studies evaluating probiotic strains

Study	Probiotics	Main outcomes
Pregnancy and lactation		
Asemi et al.[131]	Lactobacillus acidophilus La5 Bifidobacterium animalis BB12	↓ C-reactive protein
Asemi et al.[132]	L. acidophilus La5 B. animalis BB12	↑ Erythrocyte glutathione levels
Dugoua et al.[133]	Lactobacillus Bifidobacterium	No effects on birth weight, gestational age or incidence of C-section
Arroyo et al.[43] Jiménez et al.[44]	Lactobacilli from breast milk	↓ Mastitis during lactation
Allergy		
Vliagoftis et al.[134]	B. longum L. acidophilus Bacillus clausii L. paracasei L. casei L. rhamnosus	↓ Symptom severity of allergic rhinitis and medication use
Kuitunen et al.[135]	L. rhamnosus GG L. rhamnosus LC705 B. breve Bb99 Propionibacterium freudenreichii ssp shermanii JS	↓ Hb in infants Negative correlation between Hb values at 6 months and faecal calprotectin at age 3 months
Martínez-Cañavate et al.[136]	L. gasseri CECT5714 L. coryniformis CECT5711	↓ Plasma Ig E, ↑ mucosal Ig A ↑ CD4 + /CD25 + T cells ↑ Natural killer cells
Boyle et al.[138]	L. rhamnosus GG	No benefit in the treatment of eczema in children Risk of adverse effects
Lee et al.[139]	L. rhamnosus GG	Effective in prevention but not treatment of pediatric atopic dermatitis
Intestinal-related diseases		
Olivares et al.[137]	L. gasseri CECT5714 L. coryniformis CECT5711	Improvement in intestinal habits
Allen et al.[140]	L. casei strain GG Saccharomyces boulardii Enterococcus LAB SF68	↓ Duration and ↓ stool frequency in acute infectious diarrhoea
Johnston et al.[141]	Bacillus spp. Bifidobacterium spp. Lactobacillus spp. Lactococcus spp. Leuconostoc cremoris Saccharomyces spp. Streptococcus spp.	Protective effect in preventing antibiotic-associated diarrhoea
Bernaola Aponte et al.[142]	Lactobacilli Bifidobacteria Lactococci Saccharomyces, etc	↓ Duration and ↓ stool frequency in persistent diarrhoea
Alfaleh et al.[143]	Mainly lactobacilli	↓ Incidence and mortality in necrotising enterocolitis
Braga et al.[144]	L. casei B. breve	Benefit on the occurrence of necrotising enterocolitis Improvement in intestinal motility
Sang et al.[145]	B. bifidum	↑ Remission rate and ↓ recurrence rate of ulcerative colitis
Mimura et al.[146]	VSL#3	Effective in maintaining antibiotic-induced remission in patients with pouchitis for 1 year
Kühbacher et al.[147]	VSL#3	↑ Total number of intestinal bacteria in pouchitis ↑ Richness and diversity of the bacterial microbiota, especially the anaerobic microbiota Repression in fungal microbiota
Doherty et al.[148]	VSL#3 Lactobacillus rhamnosus GG L. johnsonii LA1	No effect

Table 1. *Continued*

Study	Probiotics	Main outcomes
McFarland & Dublin[149]	Mainly lactobacilli and bifidobacteria	Improvement in IBD symptoms ↓ Abdominal pain
Gawrońska *et al.*[150]	*L. rhamnosus* GG	↓ Frequency but not the severity of pain in children with IBD
Bausserman & Michail[151]	*L. rhamnosus* GG	↓ Incidence of abdominal distention in children with IBD
Enck *et al.*[152]	*Enterococcus faecalis* *Escherichia coli*	↓ Typical symptoms of IBD
Chronic liver disease Liu *et al.*[153]	*Bacillus bifidus* *L. acidophilus* *L. bulgaricus* *S. thermophilus*	↓ *E. coli* count, ↓ intestinal flora imbalance Improved symptoms and signs (debilitation, food intake, appetite, abdominal distension and ascitic fluid)
Aller *et al.*[154]	*L. bulgaricus* *S. thermophilus*	Improved liver aminotransferases in NAFLD patients
Acute pancreatitis Zhang *et al.*[155]	*L. plantarum* 299 *Pediococcus pentosaceus* *Leuconostoc mesenteroides* *L. paracasei* *L. plantarum*	↓ Length of hospital stay
Sharma *et al.*[156]	*L. acidophilus* *B. longus* *B. bifidum* *B. infantalis*	No effects
Type 2 diabetes Ejtahed *et al.*[157]	*L. acidophilus* La5 *B. lactis* Bb12	↓ Total cholesterol, LDL-C and atherogenic indices
AIDS Hummelen *et al.*[158]	*L. rhamnosus* GR-1 *L. reuteri* RC-14	No impact on the immune function of HIV-infected women who were naïve to anti-retroviral treatment
Trois *et al.*[159]	*B. bifidum* *S. thermophilus*	Preservation of the immune function of HIV-infected children
Anukam *et al.*[160]	*L. rhamnosus* GR-1	Preservation of the immune function of women naïve to anti-retrovirals
Urinary tract infections Stapleton *et al.*[161]	*L. crispatus*	↑ Vaginal colonisation, ↓ recurrent urinary tract infections
Respiratory infections Hao *et al.*[162]	*L. casei* DN-114 001	↓ Episodes of acute upper respiratory infections ↓ Antibiotic use
Siempos *et al.*[163]	Lactobacilli *P. pentosaceus* *Leuconostoc mesenteroides*	↓ Incidence of ventilator-associated pneumonia
Spondyloarthritis Jenks *et al.*[164]	*S. salivarius* *B. lactis* *L. acidophilus*	No benefit over placebo

↓, Decrease; ↑, increase; LAB, lactic acid bacteria; IBD, inflammatory bowel disease; NAFLD, non-alcoholic fatty liver disease.

have a beneficial effect in AR by reducing symptom severity and medication use.

In a study examining the effect of pre- and probiotics on the prevention of atopic disease, Kuitunen *et al.*[135] conducted a randomised study of 1223 eligible mothers carrying a child with a high risk for allergy (at least one parent with doctor-diagnosed asthma, AR or atopic eczema). Each subject received twice daily a probiotic combination of *L. rhamnosus*

GG, *L. rhamnosus* LC705, *B. breve* Bb99 and *Propionibacterium freudenreichii ssp shermanii* JS or placebo 4 weeks before delivery. Their infants received the same probiotics and 0·8 g of a galacto-oligosaccharide or placebo once daily from birth until 6 months of age. The children were observed until 2 years of age for the development of any allergic disease. Blood samples were obtained from ninety-eight infants at 6 months and 658 children at 2 years of age to measure

the haematologic values. Faecal samples were collected at 3 and 6 months of age to measure immunologic development by the expression of calprotectin, α-1-antitrypsin, TNF-α and IgA. At 6 months of age, the infants in the probiotic group had significantly lower Hb values than the placebo group. A significant negative correlation emerged between the Hb values at 6 months of age and the expression of faecal calprotectin at 3 months of age. The hematologic values in both groups were similar at 2 years of age.

Martínez-Cañavate et al.[136] evaluated the immunological effects of two probiotic strains, L. gasseri CECT5714 and L. coryniformis CECT5711, in children suffering with allergies. Olivares et al.[137] previously described a double-blinded, randomised, placebo-controlled comparative study with forty-four allergic children, who were randomly distributed into two groups: a yoghurt group and a probiotic group. In the present study, intestinal and immunological parameters were measured in faecal and blood samples. The consumption of the probiotic product induced a significant decrease in the level of IgE in the plasma and an increase in CD4 + / CD25 + T regulatory cells. The decrease in IgE was accompanied by a significant increase in mucosal IgA. Changes in other effector cells potentially involved in allergic reactions, such as eosinophiles, basophiles or other IgE + cells, were not detected. The consumption of the probiotic product also induced significant changes in the innate response, as a significant increase in natural killer cells was detected.

No evidence suggests that probiotics are an effective treatment for eczema in children; probiotic treatment carries a small risk of adverse events (infections and bowel ischaemia) and does not show any benefit in comparison with the placebo[138]. A meta-analysis of six prevention and four treatment double-blind, randomised, placebo-controlled clinical trials in children with an age ranging from 0 to 13 years indicated that present evidence favours the use of probiotics for the prevention but not the treatment of paediatric atopic dermatitis[139]. There was a 61 % risk reduction associated with the use of prenatal and/or postnatal probiotics for paediatric atopic dermatitis prevention. An additional analysis, which excluded the single study using a postnatal protocol, revealed a lower relative risk ratio. This result suggests that a prenatal component might be clinically important for maximising the prophylactic potential of probiotics. In terms of treatment, the summary effect size derived for both intergroup and intragroup differences failed to show any statistical significance.

Intestinal-related diseases

Intestinal function. Olivares et al.[137] investigated the effect of a fermented product containing two probiotic strains, L. gasseri CECT5714 and L. coryniformis CECT5711, on several blood and faecal parameters related to intestinal function in the host. A total of thirty healthy volunteers were randomly distributed into two groups, one receiving a standard yoghurt and the other a similar dairy fermented product in which the L. delbrueckii subsp. bulgaricus yoghurt strain had been replaced by a combination of the probiotic strains L. gasseri CECT5714 and L. coryniformis CECT5711. The volunteers

that received the probiotics reported no adverse effects, and the strains could be isolated from their faeces at a relatively high level. In fact, the concentration of faecal LAB significantly increased in the probiotic group. Additionally, the oral administration of the probiotics led to an improvement in several parameters, such as the production of SCFA, faecal moisture and frequency and volume of the stools. As a result, the volunteers assigned to the probiotic group perceived a clear improvement in their intestinal habits[137].

Infectious diarrhoea. A Cochrane review on the efficacy of probiotics for treating infectious diarrhoea, including both adults and children, evaluated sixty-three studies with a total of 8014 participants. No adverse events were attributed to probiotic intervention. The use of probiotics reduced the duration of diarrhoea, although the size of the effect varied considerably between studies. The average of the effect was significant for the mean duration of diarrhoea (lasting ≥4 d) and stool frequency on day 2. The authors concluded that, when used alongside rehydration therapy, probiotics appear to be safe and have clear beneficial effects in shortening the duration and reducing stool frequency in acute infectious diarrhoea[140].

Antibiotic-associated diarrhoea. A 2011 Cochrane review meta-analysis evaluated the results of sixteen randomised, parallel, placebo-controlled trials that investigated antibiotic-associated diarrhoea in children (0–18 years of age) receiving antibiotics[141]. Treatment with probiotics was compared with treatment with placebo, active alternative prophylaxis or no treatment, and the incidence of diarrhoea secondary to antibiotic use was measured. The trials included treatment with Bacillus spp., Bifidobacterium spp., Lactobacillus spp., Lactococcus spp., Leuconostoc cremoris, Saccharomyces spp. or Streptococcus spp., individually or in combination. Despite the heterogeneity in probiotic strain, dose and duration, and the quality of the study, the overall evidence suggests a protective effect of probiotics in preventing antibiotic-associated diarrhoea.

Persistent diarrhoea. The evidence suggesting that probiotics might be effective in treating persistent diarrhoea in children is scarce. Bernaola Aponte et al.[142] reviewed RCT comparing a specified probiotic agent with placebo or no probiotic in children with persistent diarrhoea. In all, four trials, with a total of 464 participants, were included in this meta-analysis. Treatment with probiotics reduced the duration of persistent diarrhoea in two trials. Similarly, the stool frequency was reduced with the use of probiotics in two trials. One trial reported a shorter hospital stay, which was significant, but the numbers were small. No adverse events were reported.

Necrotising enterocolitis. Alfaleh et al.[143] performed a meta-analysis with sixteen randomised or quasi-RCT that involved 2842 preterm infants of <37 weeks gestational age and/or weighing <2500 g at birth. These trials were highly variable with regard to enrolment criteria (i.e. birth weight and gestational age), baseline risk of NEC in the control groups, timing, dosing, probiotics formulations and feeding regimens. The data regarding extremely low birth weight infants could not be extrapolated. Enteral probiotic supplementation significantly reduced the incidence of severe NEC (stage II or more) and mortality. There was no evidence

of a significant reduction of nosocomial sepsis. Moreover, there was no evidence of systemic infection with the use of probiotics in these trials. The authors concluded that enteral supplementation with probiotics prevents severe NEC, although more studies are needed to assess the efficacy of probiotic use in extremely low birth weight infants and assess the most effective formulation and dose to be utilised.

Braga *et al.*[144] evaluated the effect of a combined supplementation of *L. casei* and *B. breve* in preterm infants with low birth weight on the occurrence of NEC as a primary outcome. The use of probiotics had a beneficial effect on the occurrence of NEC at stage ≥2 using Bell's criteria and was associated with an improvement in intestinal motility based on the time required to reach full enteral feeding.

Ulcerative colitis. Probiotic treatment is effective in maintaining remission in ulcerative colitis[145]. A total of thirteen RCT were reviewed. Compared with the non-probiotics group, the remission rate for ulcerative colitis patients who received probiotics was 1·35 (95% CI 0·98, 1·85). Compared with the placebo group, the remission rate of ulcerative colitis patients that received probiotics was 2·00 (95% CI 1·35, 2·96). During the course of treatment, patients who received probiotics for less than 12 months showed a remission rate of 1·36 (95% CI 1·07, 1·73) compared with the group treated with non-probiotics. Compared with the non-probiotics group, the recurrence rate of ulcerative colitis in patients that received probiotics was 0·69 (95% CI 1·01, 2·47). The recurrence rate was 0·25 (95% CI 0·12, 0·51) in the mild-to-moderate group that received probiotics compared with the group that did not receive probiotics. The group that received *B. bifidum* treatment had a recurrence rate of 0·25 (95% CI 0·12, 0·50) compared with the non-probiotics group.

Pouchitis is a major complication after ileal pouch anal anastomosis in patients with ulcerative colitis. Mimura *et al.*[146] showed that a single daily high dose (6 g) of probiotic VSL#3 was effective in maintaining antibiotic-induced remission in patients with pouchitis for 1 year. The remission was maintained for 1 year in 85% of patients in the VSL#3 group compared with 6% of patients in the placebo group. In a more recent paper, patients with pouchitis in remission that had been induced by antibiotic therapy were recruited to receive either the VSL#3 probiotic compound or placebo for the maintenance of remission[147]. Biopsies were obtained before and 2 months after the initiation of VSL#3 or placebo treatment. Therapy with VSL#3 increased the total number of intestinal bacterial cells and the richness and diversity of the bacterial microbiota, especially the anaerobic microbiota, whereas the fungal flora was repressed. In contrast, patients who relapsed while receiving placebo showed a reduced diversity of the mucosal microbiota.

Crohn's disease. Doherty *et al.*[148] recently reviewed trials comparing antibiotics or probiotics with placebo in the prevention of endoscopic or clinical recurrence of Crohn's disease following surgical resection. A total of seven studies were identified as suitable for inclusion (two comparing antibiotics with the placebo and five comparing probiotics with the placebo). Probiotic administration was not associated with any significant difference in the risk of recurrence compared with the placebo.

Irritable bowel syndrome. Irritable bowel syndrome (IBS) is a chronic condition affecting 3–25% of the population for which no curative treatment is available. Accordingly, therapy is aimed at reducing symptoms. Because an alteration of the normal intestinal microbiota has been observed in IBS, probiotics were considered to be useful in reducing symptoms. McFarland & Dublin[149] reviewed twenty trials that included a total of 1404 subjects. Probiotic use was associated with improvements in global IBS symptoms compared with the placebo. Probiotics were also associated with less abdominal pain.

Gawrońska *et al.*[150] investigated the efficacy of *L. rhamnosus* GG for treating functional dyspepsia, IBS or functional abdominal pain in children. These authors found that *L. rhamnosus* GG reduced the frequency but not the severity of pain in children with IBS.

In contrast to these findings, the administration of *L. rhamnosus* GG to fifty children (6–20 years) with IBS for 6 weeks was not superior to the placebo in relieving abdominal pain. There was no difference in the other gastrointestinal symptoms, except for a lower incidence of perceived abdominal distension[151].

Treatment of IBS with the bacterial lysate of *Enterococcus faecalis* and *E. coli* was effective and superior to the placebo in reducing the typical symptoms of IBS in patients treated by general practitioners[152]. In all, 297 patients with IBS were treated for 8 weeks with this bacterial lysate or a placebo, in a double-blinded, randomised fashion. The responders had at least a 50% decrease in the global symptom score, and the abdominal pain score was ≥1 visit during treatment. The responder rate in global symptom score to the probiotics was 102/149 (68·5%) compared with the placebo rate of 56/148 (37·8%; *P*<0·001), the improvement in abdominal pain score was 108/149 (72·5%) and 66/148 (44·6%), respectively (*P*=0·001). The number-needed-to-treat was 3·27 for global symptom score and 3·59 for abdominal pain score. The Kaplan–Meier analysis revealed an average response time of 4–5 weeks for active treatment and more than 8 weeks for treatment with the placebo (*P*<0·0001).

Chronic liver disease. Patients with chronic liver disease generally have an intestinal microbiota imbalance that is related to the development and worsening of the disease. Liu *et al.*[153] conducted a randomised, placebo-controlled trial, pre-test/post-test controlled group design. Patients were randomised to an experimental group (forty-one patients) or a control group (forty patients). Patients in the experimental group were given probiotic yoghurt containing *Bacillus bifidus*, *L. acidophilus*, *L. bulgaricus* and *S. thermophilus*. The subjects in the control group had meals only and were not provided with the probiotic yoghurt. After intervention, the experimental group had a lower *E. coli* count and a reduced intestinal microbiota imbalance. A comparison of the experimental and control groups after the intervention showed that the former had improved symptoms and signs, including a significant improvement in debilitation, food intake, appetite, abdominal distension and ascitic fluid.

Aller et al.[154] showed that the ingestion of a tablet of 500 million *L. bulgaricus* and *S. thermophilus* improved liver aminotransferase levels in patients with non-alcoholic fatty liver disease.

Acute pancreatitis. Zhang et al.[155] reviewed all relevant RCT that studied the effects of pre-, pro- or synbiotics in patients with acute pancreatitis. A total of seven randomised studies with 559 patients were included. Pre-, pro- or synbiotic treatment showed no influence on the incidence of postoperative infections, pancreatic infection, multiple organ failure and systemic inflammatory response syndrome. There were also no significant differences in the length of antibiotic therapy and mortality. However, pre-, pro- or synbiotic treatment was associated with a reduced length of hospital stay.

Sharma et al.[156] investigated the role of probiotics on gut permeability and endotoxaemia in patients with acute pancreatitis. Patients were randomised to receive either a placebo or a mixture of *L. acidophilus*, *B. longus*, *B. bifidum*, *B. infantalis* and 25 mg of fructo-oligosaccharide. No significant trend was identified concerning the effect of probiotics on gut permeability or endotoxaemia in acute pancreatitis. However, the study was underpowered owing to premature study termination.

Type 2 diabetes. Ejtahed et al.[157] investigated the administration of probiotics in type 2 diabetic patients, who were randomised to receive either 300 g of probiotic yoghurt containing *L. acidophilus* La5 and *B. lactis* Bb12 or 300 g of conventional yoghurt for 6 weeks. Probiotic consumption caused significant decreases in total cholesterol, LDL-C and the atherogenic indices total cholesterol:HDL-C ratio and LDL-C:HDL-C ratio compared with the controls.

AIDS. HIV-infected women who were naïve to antiretroviral treatment were randomised to receive oral capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or placebo twice daily for 25 weeks. The CD4 count and immune markers (IgG, IgE, IFNγ and IL-10) were measured at baseline and during follow-up. Probiotics had no impact on the immune function in the present study[158].

Other trials have shown a preservation of the immune function with the use of probiotics among non-responsive children or those treated with the anti-retrovirals *B. bifidum* and *S. termophilus* in Brazil[159] and among women naïve to anti-retrovirals who were treated with *L. rhamnosus* GR-1 in Nigeria[160].

Urinary tract infections. Urinary tract infections (UTI) are common among women and frequently recur. The depletion of vaginal lactobacilli is associated with UTI risk, which suggests that repletion might be beneficial. Stapleton et al.[161] conducted a double-blind placebo-controlled trial of a *L. crispatus* intravaginal suppository probiotic for the prevention of recurrent UTI in premenopausal women. Recurrent UTI occurred in 15 % of women receiving probiotics compared with 27 % of women receiving placebo (relative risk, 0·5; 95 % CI 0·2, 1·2). High-level vaginal colonisation with *L. crispatus* throughout follow-up was associated with a significant reduction in recurrent UTI only in the group that received probiotics.

Respiratory infections. Hao et al.[162] performed a meta-analysis that included ten RCT comparing probiotics with placebo to prevent acute upper respiratory tract infections. Probiotics were more effective than the placebo in reducing the number of participants experiencing episodes of acute upper respiratory tract infections, the rate ratio of episodes of acute upper respiratory tract infections and reducing antibiotic use.

A meta-analysis of five RCT showed that the administration of probiotics is associated with lower incidence of ventilator-associated pneumonia compared with the placebo[163].

Spondyloarthritis. Jenks et al.[164] studied the effect of an orally administered probiotic on disease activity, fatigue, quality of life and intestinal symptoms in patients with active spondyloarthritis. In the present randomised placebo-controlled trial, the probiotic combination did not demonstrate significant benefit over the placebo.

Conclusions and future directions

Lactobacilli and bifidobacteria are the genera most frequently used as probiotics. Traditional fermented products and the breast milk, GIT and faeces of human subjects are the primary sources of these microorganisms. Probiotics are isolated by culture in selective media. Currently, the identification of probiotic strains is facilitated by the sequencing of their *16S RNA* genes. Prior to their evaluation, probiotics must be characterised using the following criteria: (1) the capacity to resist extremely low pH, gastric and intestinal enzymes and bile salts, (2) the capacity to adhere to intestinal epithelial cells, (3) antimicrobial activity and (4) safety. The evaluation of probiotics can be conducted at the preclinical (cell and animal models) and clinical levels. Among the latter, the most reliable studies to assess the therapeutic benefits of any probiotic strain are randomised, placebo-controlled trials.

Probiotics have been shown to promote a variety of biological effects in a number of physiological conditions and pathologies, including allergy, intestinal and liver diseases, urinary and upper respiratory infections, AIDS and metabolic diseases. These effects are strain specific and are primarily mediated through changes in the faecal microbiota and immune modulation. RCT concerning the appropriate clinical evaluation of probiotics, with an adequate and statistically sufficient number of subjects related to the main outcome variables, should be performed in a variety of diseases. In addition, multi-centre and replicate studies are necessary to evaluate the actual role of probiotics in the amelioration of symptoms for many diseases. The number of studies concerning the mechanism of probiotics in cell and animal models is scarce. Apparently, many probiotics are able to modulate both the innate and adaptive immune responses; however, the molecular basis of these effects remains unknown.

Acknowledgements

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